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Identification of a Human Anti-CD55 Single-Chain Fv by Subtractive Panning of a Phage Library Using Tumor and Nontumor Cell Lines

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ABSTRACT

A large naïve human single-chain (sc) Fv phage library was used to search for tumor-associated antigens by panning with a lung adenocarcinoma cell line, 1264, and counter-selecting with a nontumor bronchial epithelial cell line, BEAS-2B. After three rounds of subtractive panning, 239 of 673 clones analyzed bound selectively to 1264 tumor cells in a phage ELISA. Diversity analysis of these tumor-selective clones by *Bst*NI fingerprinting and nucleotide sequencing revealed 14 distinct scFv fragments. Four clones bound selectively to 1264 over BEAS-2B cells when analyzed by a more discriminating flow cytometric assay using scFv. Moreover, these clones showed only limited cross-reactivity to several primary human cell lines. One clone, LU30, also cross-reacted strongly with the lung adenocarcinoma line, A549. The LU30 antigen was identified as decay-accelerating factor (CD55) by expression cloning from a 1264 cDNA library. The mean number of decay-accelerating factor molecules on the surface of 1264 and BEAS cells used for panning and counter-selection was estimated as 75,000 ± 5,000 and 13,000 ± 10,000, respectively. Thus, phage library panning combined with expression cloning permits identification of antibodies and their cognate antigens for proteins that are differentially expressed on the surface of distinct cell populations.

INTRODUCTION

The demonstration of significant antitumor efficacy of antibodies has long been sought-after in the clinic and recently obtained using naked chimeric (1–4) and humanized (5) antibodies, as well as with radiolabeled murine antibodies (6–9). Indeed, a chimeric anti-CD20 antibody (10) and a humanized anti-HER2 antibody (11) were approved recently by the United States Federal Drug Administration for the treatment of non-Hodgkin's lymphoma and metastatic breast cancer, respectively. These successes with antitumor antibodies in patients has led to renewed interest in the identification of novel tumor-associated antigens suitable for antibody targeting.

The traditional approach to obtaining tumor-specific antibodies has been to immunize mice with tumor cells and to screen the resultant monoclonal antibodies for their binding specificity. Unfortunately, tumor-binding antibodies obtained in this way often cross-react with many normal cells, which may interfere with their clinical use. Ideally, one would like to select rather than screen for antibodies that bind selectively to tumor. The advent of antibody fragment display on phage (12) and the development of large (>6×10^9 clones) phage display libraries (13–15) offer a potential way of doing this by panning using tumor cells and counter-selecting using nontumor cells. An additional advantage of antibody phage is that, unlike hybridoma technology, it is readily possible to obtain antibodies binding antigens that are highly conserved between mouse and man (16).

Naïve antibody phage libraries have proved to be a rapid and general method for identifying antibodies binding to purified antigens (13–16). In contrast, panning cellular targets with antibody phage has proved much more difficult because of the much lower effective antigen concentration, greater antigen complexity, and the tendency of phage to bind nonspecifically to cells. Nevertheless, significant progress has been made, allowing the identification of antibodies against cell surface antigens (17–23). Indeed, melanoma-specific antibodies have been identified by selecting for antibody phage that bind to melanoma cells, but not melanocytes, using antibody phage libraries constructed from human donors immunized with their own tumor cells (21–23).

We have extended the use of antibody phage libraries for panning on live tumor cells by using a large naïve library (14). This obviates the need for creating custom libraries from immunized donors (21, 23). In addition, live rather than fixed cells (21–23) were used for screening to facilitate the identification of antibodies that bind to native rather than denatured antigens. This was done to facilitate subsequent expression cloning of corresponding antigen, as well as enhance the therapeutic potential of antibodies obtained. Indeed, we cloned the antigen corresponding to a scFv[4] fragment identified with significant tumor selectivity.

MATERIALS AND METHODS

Cell Lines. The lung adenocarcinoma line 1264 was kindly provided by Dr. A. Gazdar (Simmons Cancer Center, University of Texas-Southwestern, Dallas, TX) and grown in RPMI 1640 supplemented with 10% (v/v) FBS. The lung adenocarcinoma cell lines SKLU1, A549, and CALU6 were obtained from American Type Culture Collection (Manassas, VA) and grown in a 1:1 mixture of RPMI 1640 and DMEM supplemented with 10% (v/v) FBS. The BEAS-2B cell line, constructed by SV40 transformation of human bronchial epithelial cells, was obtained from American Type Culture Collection, as was CCD19LU, a fibroblast-like cell line isolated from normal human lung. Both BEAS-2B and CCD19LU cells were cultured in RPMI/DMEM/FBS. NHBE 4683 and NHEK 4021 are primary cell lines (Clonetics, San Diego, CA) that were cultured in the serum-free media, BEGM and KGM (Clonetics), respectively. NHBE 4683 and NHEK 4021 lines were used for subtractive panning or analysis between the third and fourth passage. All cell lines were grown adherently and detached with 2.5 mM EDTA in PBS before use.

Live Cell Panning with scFv Phage. An aliquot containing 2.5×10^{12} cfu phage, from a large human scFv phage library (14) was blocked with 500 μl of RPMI containing 10% (v/v) FBS, 1 mM phenylmethylsulfonyl fluoride, and 2.5 mM EDTA to reduce nonspecific binding to cell surfaces. The blocked phage were added to 1×10^6 BEAS-2B cells in 500 μl of RPMI/DMEM/FBS and mixed gently for 30 min at ~20°C. Cells were then pelleted, at this and subsequent panning steps, by centrifugation at 500 × *g* for 5 min at 4°C. The phage-containing supernatant was used to resuspend a fresh pellet of 1×10^6 BEAS-2B cells and was incubated for 30 min at ~20°C, followed by pelleting the cells. After repeating this counter-selection step, the resultant "subtracted" phage supernatant was incubated with 5×10^6 1264 cells for 1 h at ~20°C with gentle mixing. The cells were pelleted and washed three times with PBS. The cell-bound phage were eluted with 0.5 ml of PBS containing 100 mM citric acid (pH 2.2) for 10 min and then neutralized with 0.5 ml of 1.0 M Tris-HCl (pH 7.5).

Escherichia coli strain TG1 (New England Biolabs, Beverly, MA) in mid-logarithmic growth phase (A_{550} = 0.4–0.8) was infected with the eluted phage and plated on 2YT agar containing 2% (w/v) glucose and 50 μg/ml

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[4] The abbreviations used are: scFv, single-chain Fv; DAF, decay-accelerating factor; FBS, fetal bovine serum; cfu, colony-forming unit; NHBE, normal human bronchial epithelial; NHEK, normal human epidermal keratinocyte.

carbenicillin (2YTGC). The resultant colonies were propagated and used to prepare phage (24). An aliquot containing ~1 × 10^{12} cfu phage was used for a second round of panning, consisting of five counter-selections using 1 × 10^6 BEAS-2B cells, followed by selection using 1 × 10^7 1264 cells for ~15 h at ~20°C. After 10 washes with PBS, the cell-bound phage were eluted and then neutralized as in the first round of panning. The eluted phage were propagated, and a third round of panning was performed using 1.0 × 10^{12} cfu phage and the second round protocol.

Cell ELISA with Phage. The scFv-phage were compared in their binding to live tumor and nontumor cells by ELISA as a primary screen of their binding specificity. After the third round of panning, a culture of TG1 was infected with the eluted phage and plated on 2YTGC. Clones for analysis were transferred into 96-well plates with 100 μl of 2YT media containing 2% (w/v) glucose and carbenicillin (100 μg/ml) and grown for ~18 h with agitation at 30°C. Glycerol (50 μl 50%, v/v) was added to each well of these master plates before storage at −70°C.

Replicas of the master plates were prepared, and scFv-phage were induced by superinfection with M13KO7 helper phage and overnight incubation at 30°C (24). The plates were centrifuged (300 × *g*, 5 min, 4°C) at this and subsequent cell ELISA steps to pellet the bacteria, and 100 μl of scFv-phage-containing supernatants were transferred to 96-well plates containing 100 μl of 6% (w/v) BSA in PBS/well. Blocked scFv-phage supernatants (100 μl) were added to parallel plates containing either 1 × 10^5 1264 or BEAS-2B cells/well (1 h, 4°C, gentle agitation). The plates were centrifuged, and supernatants were aspirated without disturbing the pellets. The cells were washed twice by resuspension in 200 μl of 4% (v/v) FBS in PBS (ELISA buffer) at 4°C, followed by centrifugation. Pellets were then resuspended in 100 μl of ELISA buffer containing a 1:5,000 dilution of horseradish peroxidase conjugated to a sheep anti-M13 polyclonal (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated for 20 min at 4°C. Cells were centrifuged and washed three times in ELISA buffer. Cell pellets were resuspended in 100 μl of TMB reagents (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) and developed for ~10 min before quenching with 100 μl of 1 M phosphoric acid. The ELISA plates were read (A_{450}–A_{650}) using a Spectramax 340 microtiter plate reader (Molecular Devices, Sunnyvale, CA), and data were analyzed using a spreadsheet program (Microsoft Excel 5.0a).

Flow Cytometry with Phage and scFv. Culture supernatants containing scFv phage were incubated with cells and washed, as described above, for the cell ELISA with the following modifications. The anti-M13 polyclonal antibody was used in unconjugated form. After washing, the cells were incubated for 20 min at 4°C with an R-phycoerythin-conjugated F(ab')$_2$ fragment from a donkey antisheep IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:200 in ELISA buffer, followed by three washes and resuspension in 0.5 ml of ELISA buffer. Cells were analyzed using a FACScan flow cytometer (Beckton and Dickinson, Mountain View, CA).

For cytometric analysis with scFv fragments, 1 × 10^5 cells in ELISA buffer were incubated for 1 h at 4°C with 3 μg/ml scFv fragment. The cells were washed twice by centrifugation and resuspension in ELISA buffer. Cell pellets were then resuspended in 100 μl of ELISA buffer containing 1 μg/ml BMG-His1 (Boehringer Mannheim, Indianapolis, IN), the antihexahistidine monoclonal antibody. Cells were washed three times in ELISA buffer before resuspension in 100 μl of ELISA buffer containing a 1:200 dilution of a F(ab')$_2$ fragment of a goat antimouse IgG conjugated with FITC (Jackson Immunoresearch Laboratories). After three additional washes, the cells were analyzed by flow cytometry.

Quantitation of Cell Surface DAF. The mean number of DAF molecules/cell was estimated by flow cytometry using a FITC-labeled antibody in comparison with FITC-conjugated beads, using the method of Christensen and Leslie (25), with the following modifications. Murine anti-DAF monoclonal antibody 1A10 (250 μg; Genentech Inc.) in 50 mM sodium carbonate (pH 8.5) was incubated with 12 μg of *N*-hydroxysuccinimidyl-fluorescein (Pierce Chemical Co., Rockford, IL) for 2 h at 20°C, followed by extensive dialysis against PBS. The molar ratio of FITC to protein was determined from the absorbance at 280 nm and 492 nm (25). Cells were incubated with varying levels of the FITC-labeled anti-DAF antibody to achieve saturation and then prepared for flow cytometry, as above.

Clone Diversity Analysis. The diversity of antigen-positive clones was analyzed by PCR-amplification of the scFv insert using the primers fdtetseq and PUC19 reverse (16), digestion with *Bst*NI (24), and analysis by PAGE. Comparison of *Bst*NI fingerprints was facilitated by digitization of the gel data using an AlphaImager (Alpha Innotech Corp., San Leandro, CA) and analysis using ProRFLP version 2.34 (DNA ProScan, Nashville, TN). Up to 10 clones/*Bst*NI fingerprint were then cycle-sequenced using rhodamine-labeled dideoxy chain terminators (Applied Biosystems, Foster City, CA), using M13 reverse (New England Biolabs) and mycseq10 primers (16). Samples were analyzed using Applied Biosystems Automated DNA Sequencers (models 373 and 377), and sequence data were analyzed using the program Sequencher version 3.1 (Gene Codes Corp., Ann Arbor, MI).

scFv Production. Selected scFv clones were transformed into *E. coli* strain 33D3 (W3110 *tonA ptr3 phoA*ΔE15 *lacI*^{q} *lacL8 degP kanR*; Ref. 26), and cultured for 18 h at 30°C in 2YT media containing 0.2 mM isopropyl-β-D-galactopyranoside to induce scFv expression. Periplasmic extracts were prepared by resuspending a bacterial pellet from a 500-ml culture in 10 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 M NaCl, 25 mM imidazole, 0.2 mg/ml hen egg white lysozyme, and 1 mM phenylmethylsulfonyl fluoride. After incubation for 1 h at 4°C, the debris was removed by centrifugation. Supernatants were filtered (0.2 μm) and, the His-tagged scFv fragments were purified by immobilized metal affinity chromatography using Ni^{2+}-nitrilotriacetic acid agarose (Qiagen, Valencia, CA). The scFv fragments were eluted with 250 mM imidazole in PBS, then dialyzed into PBS, flash frozen, and stored at −70°C. Clones LU1, LU4, LU13, LU20, and LU30 were grown to high cell density in the fermenter, as described previously (27). scFv fragments were purified from 2 g of fermentation pastes, as for cell pellets from shake flasks.

cDNA Library Construction. Total cellular RNA was purified from guanidine thiocyanate homogenates from 6 g of cultured 1264 cells (28). mRNA was isolated from the total RNA using oligo-d(T) cellulose (Collaborative Research, Bedford, MA; Ref. 29). Oriented cDNA transcripts were prepared from 5 μg of poly(A)+ mRNA using the SuperScript Plasmid System (Life Technologies, Inc., Gaithersburg, MD), fractionated by electrophoresis on a 5% polyacrylamide gel, and size selected in the ranges of 0.6–2.0 kb and >2 kb. Eluted cDNAs were ligated into the *Xho*I and *Not*I sites of the mammalian expression vector pRK5 (30) and then electroporated into DH10B (Life Technologies, Inc.) cells under conditions recommended by the supplier.

Antigen Expression Cloning from cDNA Library. DNA from 10 pools of 50,000 clones each of the 0.6–2 kb and ≥2 kb cDNA libraries was prepared for expression cloning the antigens recognized by tumor-selective scFv fragments. Plasmid DNA (10 μg) from each of the 20 pools was electroporated into 2 × 10^6 COS7 cells in 180 μl of PBS using 4-mm gap cuvettes with a Gene Pulser electroporator (Bio-Rad, Hercules, CA) with an applied voltage of 300 V and a capacitance of 125 μF. After incubation for 72 h at 37°C, the COS7 cells were detached with 2.5 mM EDTA in PBS. The cells were washed and then incubated in 1 ml of growth media containing one or more purified scFv fragment (10 μg/ml each) for 1 h at 4°C. The cells were washed twice to remove unbound scFv, resuspended in 1 ml of media containing 5 μg of anti-penta-histidine antibody (Qiagen) and incubated for 1 h at 4°C. After two to three washes, the cells were resuspended in 5 ml of media and transferred to a polystyrene dish coated with a polyclonal antimouse IgG (ICN/Cappel, Aurora, OH) and allowed to bind for 1 h at 4°C. Plates were washed gently three to four times with PBS. Remaining attached cells were lysed, plasmid DNA-extracted, and amplified (31). This DNA was then electroporated into COS7 cells for additional panning. In one case, an increasing number of cells were captured during the second to fourth rounds of panning. Plasmid DNA extracted from the COS7 cells was transformed into TG1, and single colonies were picked into 96-well plates. DNA was prepared from pools of 10–20 clones each, electroporated into COS7 cells, and panned with scFv fragments, as described above. Pools of clones positive for cells binding to the Petri dishes were broken down from the *E. coli* master plates, and individual clones were tested by panning. An individual positive clone was cycle-sequenced using rhodamine-labeled dideoxy chain terminators.

Affinity Measurements. Kinetic measurements were made by surface plasmon resonance using a Biacore 1000 Biosensor (Biacore, AB Uppsala, Sweden). CM-5 chips were functionalized with 350 response units of recombinant human DAF in 10 mM sodium acetate (pH 4.6) or 8000 response units of BSA as a negative control. The DAF-derivatized chip was saturated with LU30 scFv (25–100 nM) by injecting this fragment at 10 μl/min in PBS containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20. The resultant sensorgrams were analyzed using BIAevaluation software 3.0.

RESULTS

Subtractive Cellular Panning with scFv Phage. A large human scFv-phage library (14) was used to search for novel tumor-associated antigens by panning with the lung adenocarcinoma cell line 1264 and

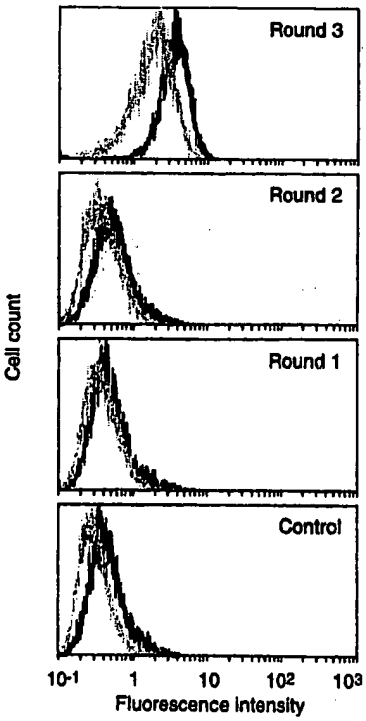

Fig. 1. Flow cytometric analysis of phage populations from Rounds 1, 2, and 3 binding to tumor cell line 1264 (*black*) used for selection and nontumor line BEAS-2B (*gray*) used for counter-selection. Also shown is a negative control phage population.

counter-selecting with the nontumor bronchial epithelial cell line BEAS-2B. Precautions were taken to maintain the integrity of membrane antigens during panning to facilitate subsequent identification of antigen by expression-cloning using isolated scFv fragments. First, live rather than fixed cells were used for panning in an attempt to preserve surface antigens in their native state. Second, cells grown adherently were detached with EDTA alone, thereby avoiding proteolytic degradation of cell surface antigens resulting from the commonly used trypsin release step.

The number of phage recovered after one, two, and three rounds of panning was 1.5×10^7, 7.0×10^5, and 4.0×10^6 cfu, respectively. The phage populations after each round of panning were analyzed by flow cytometry. The phage from the third round showed a large increase in binding to 1264 cells and a slightly smaller increase with BEAS-2B cells when compared with phage from prior rounds and unselected phage (Fig. 1). This apparent differential increase in binding to 1264 over BEAS-2B cells encouraged us to screen individual phage from the third round population for selective binding to the 1264 tumor cells.

Analysis f Clone Specificity and Diversity. The binding specificity of individual clones from the third round of panning was analyzed by ELISA using scFv-phage and live cells. The primary criteria used to assess tumor-selectivity were robust binding to 1264 cells (A_{450}–$A_{650} \geq 0.3$) and much weaker if detectable binding to BEAS-2B cells (≥10-fold lower ELISA signal). The diversity of clones satisfying these primary criteria was assessed by *Bst*NI fingerprinting of the PCR-amplified scFv fragments, and nucleotide sequencing of up to 10 clones/fingerprint pattern. A small number of clones that did not satisfy the primary criteria were also fingerprinted ($n = 29$) and sequenced ($n = 11$). Secondary criteria were then used to choose unique and apparently tumor-selective clones for further analysis: (*a*) open reading frame for scFv; and (*b*) majority of clones that share the same nucleotide sequence also satisfy the primary selection criteria.

As anticipated, the majority of clones assayed show detectable binding to 1264 cells by phage ELISA, many with limited cross-reactivity to BEAS-2B cells (Fig. 2). Of 673 clones analyzed, 239 satisfied the primary criteria for selective binding, and 197 clones could be assigned to 15 different *Bst*NI fingerprint patterns (Table 1). In the majority of cases (13 of 15), one fingerprint pattern gave rise to a single nucleotide sequence, whereas in 2 of 15 cases, two different sequences were found with indistinguishable *Bst*NI fingerprint patterns. Thus, a total of 17 scFv clones that satisfy the secondary selection criteria were identified. The two most abundant clones, fingerprints types 1 and 2, represented ~80% of the clones satisfying the secondary criteria. In contrast, the other 15 clones each represent ≤5% of the clones identified. Four of the 17 clones (LU1, LU3, LU22, and LU36) are so closely related (≥97% amino acid identity for scFv; Fig. 3*A*) that they were considered to be four variants of one distinct scFv. Thus, from the 673 clones initially screened, 14 distinct scFv clones were identified that show selective binding to 1264 cells as judged by phage ELISA. These 14 distinct scFv fragments have divergent V_H sequences (Fig. 3*B*), whereas their corresponding V_L domains are more limited in diversity (Fig. 3*C*). Indeed, many of the scFv clones isolated use identical or very closely related V_L sequences as, noted previously (14, 32). This reflects the very limited size of the light chain repertoire in the phage library.

Stringent Analysis of Clone Specificity. The 14 distinct clones that satisfied the secondary selection criteria (Table 1) were further analyzed by a more discriminating, but low, throughput flow cytometric screen using scFv fragments (representative data in Fig. 4). Four clones,

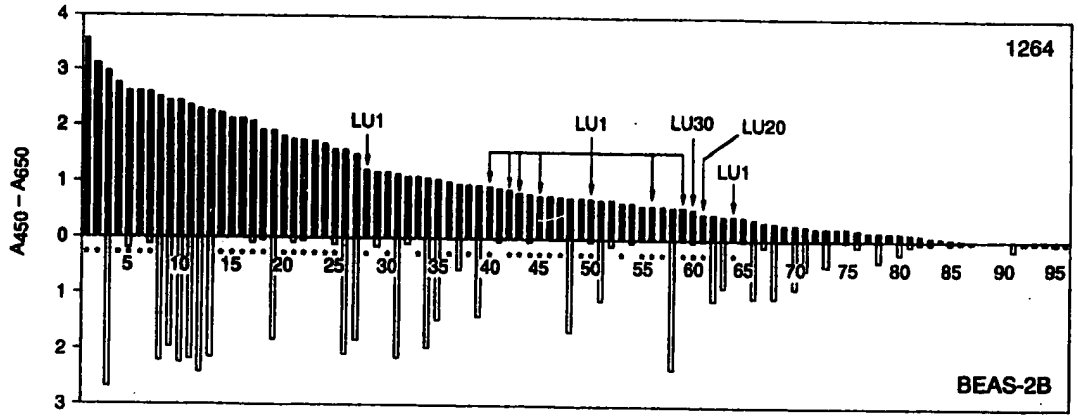

Fig. 2. ELISA screening of scFv phage binding to 1264 and BEAS-2B cells. Highlighted clones (*) satisfy the primary criteria for tumor selectivity: A_{450}–$A_{650} \geq 0.3$ for binding to 1264 cells and ≥10-fold lower signal with BEAS-2B cells. Identified clones (*arrows*) bind 1264 cells with minimal cross-reactivity to BEAS-2B cells in a stringent flow cytometric assay using purified scFv fragments (see "Results"). Data are sorted by the ELISA signal with 1264 cells and are taken from a representative 96-well plate.

Table 1 *Primary screening of scFv phage clones*

*Bst*NI fingerprint type	Number of tumor-selective clones[a]	Clone identity (number of clones sequenced)
1	110	LU4 (8)
2	49	LU1 (7)
3	10	LU20 (9)
4	7	LU13 (3), LU34 (4)[b]
5	4	LU22 (4)
6	3	LU36 (3)
7	3	LU41 (3)
8	3	LU57 (2)
9	3	LU3 (1), LU77 (2)[b]
10	2	LU30 (2)
11	1	LU7 (1)
12	1	LU71 (1)
13	1	LU100 (1)
14	1	LU60 (1)[c]
15	1	LU78 (1)

[a] Tumor-selective clones by phage ELISA: robust binding to 1264 cells ($A_{450} - A_{650} \geq 0.3$) and much weaker binding to BEAS-2B cells (≥10-fold lower signal).

[b] Clones LU13 and LU34 are predicted from their nucleotide sequences to generate identical fingerprint patterns, whereas clones LU3 and LU77 share closely related fingerprints that were not distinguishable by our electrophoretic analysis.

[c] Codon 3 in V_H is amber (TAG) that will be read through as glutamine in the *sup*E *E. coli* strain, TG1.

namely LU1, LU13, LU20, and LU30, demonstrated significant binding to 1264 cells, but minimal cross-reactivity to BEAS-2B cells. In contrast, the remaining clones (represented by LU4 in Fig. 4) showed significant cross-reactivity to BEAS-2B. Clone LU30, which gave the most pronounced binding to 1264, also gave strong staining of one of three additional lung adenocarcinoma lines tested (A549). Clones LU1, LU13, LU20, and LU30 showed minimal cross-reactivity to BEAS-2B and the primary human line NHEK 4021 (Fig. 4). Flow cytometric analysis with the primary human lines CCD-19LU and NHBE 4683 gave very similar binding between LU1, LU13, LU20, and LU30 phage as control phage, albeit with substantially higher background binding than for the other lines (data not shown).

Expression Cloning of LU30 Antigen. ScFv fragments corresponding to clones LU13, LU20, and LU30 were prepared by secretion from *E. coli* and immobilized metal affinity chromatography and used for expression cloning. Panning was performed using a mixture of these three scFv fragments, and a cDNA expression library was constructed from 1264 cells that was transiently expressed in COS7 cells. After three rounds of panning using a mixture of these three scFv fragments, efficient cell capture was demonstrated with some plasmid pools using LU30, but not LU13 and LU20 scFv fragments. Repeated panning using clones LU13 and LU20 in the absence of LU30 was also unsuccessful. Positive pools for clone LU30 were broken down first into smaller pools and then into individual clones. This led to the identification of a single clone expressing a protein that bound specifically to the LU30 scFv fragment. Nucleotide sequence analysis of this clone identified it as DAF (CD55). Binding of LU30 scFv (GenBank accession number AF117206) to 1264 cells could be competed with recombinant human DAF (Fig. 5), but not with the anti-DAF monoclonal antibody 1A10 (data not shown). Further confirmation of LU30 binding to DAF was provided by affinity measurements obtained using a BIAcore instrument: $K_d = (13 \pm 5)$ nM, $k_{on} = (3.4 \pm 1.0 \times 10^5\ M^{-1}\ s^{-1}$, $k_{off} = (4.5 \pm 1.3 \times 10^{-3}\ s^{-1}$.

Cellular DAF Levels. The mean number of DAF molecules on 1264 and BEAS-2B cell lines was estimated by quantitative flow cytometry using an anti-DAF IgG labeled with a mean number of 5.3 FITC m lecules in comparison with standards. The number f DAF molecules on the 1264 tumor cells used for panning and BEAS nontumor cells used for counter-selection was estimated as 75,000 ± 5,000 and 13,000 ± 10,000, respectively. Attempts to estimate the number of DAF sites on BEAS-2B and 1264 using this methodology with the LU30 scFv fragment were unreliable because FITC labeling of LU30 scFv impaired its binding to DAF.

DISCUSSION

We have identified four scFv fragments that bind more extensively to one or more tumor cell lines than to related nontumor cell lines by subtractive panning of live cells with a large naive antibody phage library. The cognate antigen corresponding to one scFv clone, LU30, was identified as DAF by expression cloning. DAF is expressed at ~6-fold greater levels on 1264 cells than BEAS cells used for counter-selection. Thus, the counter-selection process is not 100% efficient, permiting identification of a scFv fragment that binds to antigen that is present at much higher levels on target than control cells. This bodes well for the use of this method because cell surface antigens that are overexpressed in tumors compared with normal tissues occur frequently [*e.g.*, *HER2/neu* (33) and epidermal growth factor receptor (34)]. Antigens that are present in tumors but absent from normal tissues have been much sought after, but have, thus far, proved elusive.

The LU30 antigen DAF is a glycophosphatidylinositol-anchored protein that acts together with two other glycophosphatidylinositol-anchored proteins, CD46 and CD59, in protecting host cells from complement-mediated cell lysis (35). DAF is expressed at widely varying levels on tumor cell lines, and its overexpression correlates

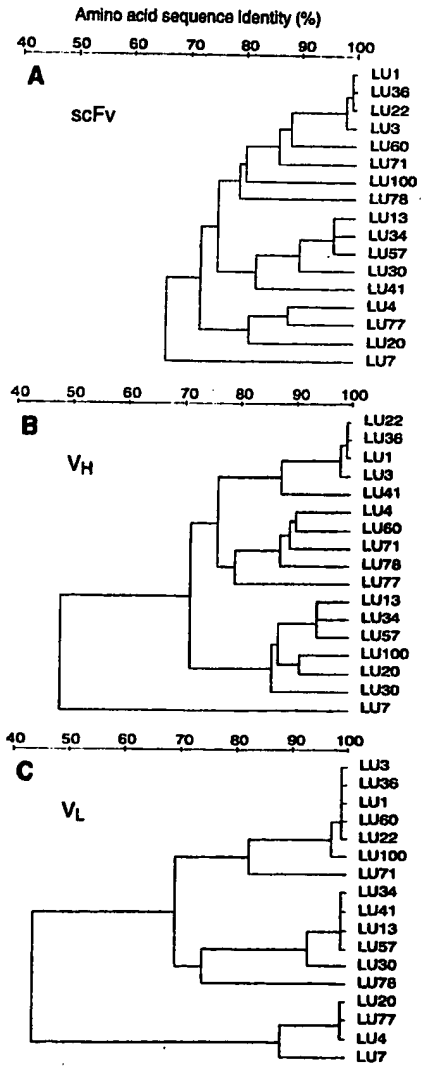

Fig. 3. Dendrograms for tumor-selective scFv satisfying primary and secondary selection criteria (see Table 1). Comparisons were made between scFv amino acid sequences (*A*), as well as their component V_H domains (*B*), and V_L domains (*C*).

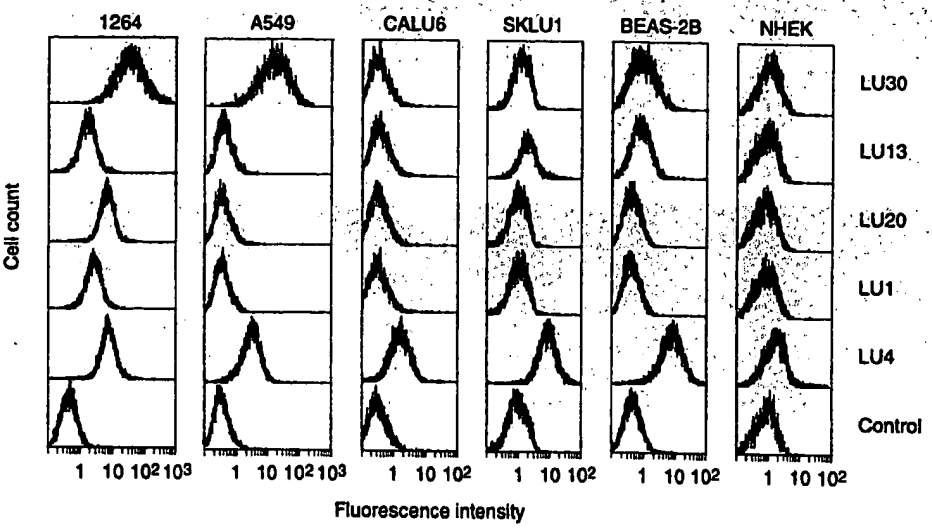

Fig. 4. Flow cytometric analysis of scFv fragments with tumor (1264, A549, CALU6, and SKLU1) and nontumor (BEAS-2B and NHEK) cell lines.

with enhanced resistance to complement-mediated cell lysis *in vitro* (36). DAF overexpression has been observed on a variety of human tumor tissues including six of nine lung adenocarcinomas and two of seven lung squamous cell carcinomas (37). Nevertheless, DAF seems poorly suited as a target for tumor immunotherapy because it is broadly expressed on the surface of normal cells, particularly those exposed to serum complement (35). Regarding normal lung tissue, DAF has been detected by immunohistochemistry on the alveolar epithelium, interstitium, and endothelium, as well as the bronchial epithelium, glands and ducts, and also blood vessels (37).

Antibody phage panning method offers a potential direct and broadly applicable route to the identification of human antibodies suitable for antitumor therapy. This strategy likely favors the identification of antibodies to highly expressed antigens, such as DAF shown here, because high antigen levels are anticipated to facilitate enrichment of cognate-scFv phage during panning. This seems desirable because high-level antigen expression may also facilitate tumor localization of antitumor antibodies *in vivo*.

Antibody phage panning could potentially identify tumor-associated antigens resulting from posttranslational modifications that differ between tumor and nontumor cells [*e.g.*, the mucin product of the *MUC1* gene is underglycosylated in many human tumors (38) exposing new epitopes for antibody targeting]. This has prompted the development of humanized anti-MUC1 antigen (39–41). Furthermore, human antibodies recognizing MUC1 on tumor cells have been identified by panning with a MUC1 peptide (42). In contrast, such posttranslational differences between tumor and nontumor cells will not be detected by powerful high throughput transcriptome and genomic methods, such as differential display (43) cDNA (44, 45) or oligonucleotide (46) microarray and serial analysis of gene expression (47–49). Transcriptome and genomic methods will also fail to detect proteins which are overexpressed in tumors despite unchanged RNA transcript levels and gene copy number, respectively.

Serial analysis of gene expression has identified significant differences in RNA transcript levels between primary human tumors and tumor cells lines (48). This raises the possibility that antibody phage panning may fail to detect tumor-associated antigens found on primary human tumors, but absent on cell lines. Conversely, antibodies may be identified that are cell line-specific as judged by failure to bind primary human tumor cells. Direct panning on primary human tumor cells is anticipated to avoid these problems, but entails as yet unaddressed technical challenges. Credence to the notion that these obstacles are surmountable is provided by reports of successful panning with peptide phage libraries *in vivo* (50–52).

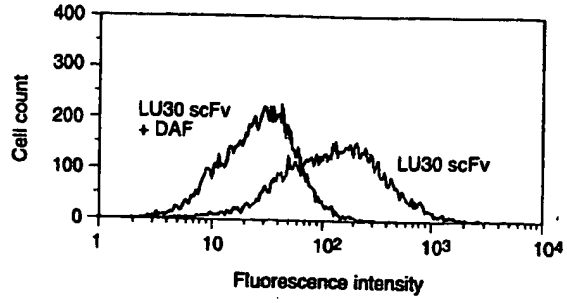

Fig. 5. Binding of LU30 scFv (3 μg/ml) to 1264 cells in the absence and presence of recombinant human DAF (30 μg/ml).

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